

*Claims
Re-numbered*

WHAT IS CLAIMED IS:

*at sec
37 CFR
1.126*

1. A method for enrichment of natural antisense messenger RNA (mRNA), comprising:

generating a population of cDNA from poly A+ mRNA in a sample of RNA using a reverse transcriptase enzyme and a polydeoxythymidine-containing oligonucleotide primer having at its 5'-end a sequence identical to an amplification primer used in a later step in the method;

incubating the population of generated cDNA to hybridize a sense cDNA from the population of cDNA with an antisense cDNA from the same population of cDNA, wherein the antisense cDNA has a single-stranded segment complementary to the sense cDNA and hybridizes thereto to form a hybrid molecule with a double-stranded segment;

treating the hybrid molecule with a DNA polymerase having a 5' to 3' polymerase activity and a 3' to 5' exonuclease activity to remove any single-stranded non-hybridized segments of the hybrid molecule from 3' to 5' and to extend the double-stranded segment of the hybrid molecule 5' to 3' over an adjacent single-stranded segment as template, thereby forming a double-stranded molecule;

amplifying the double-stranded molecule using a thermostable polymerase and an amplification primer identical to the sequence at the 5'-end of the polydeoxythymidine-containing oligonucleotide primer; and

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cloning the amplified double-stranded molecule to enrich for a natural antisense mRNA encoded by the amplified double-stranded molecule.

2. The method according to claim 1, wherein the DNA polymerase in said treating step is T4 DNA polymerase.

3. The method according to claim 1, wherein the DNA polymerase in said treating step is Platinum Pfx DNA polymerase.

4. The method according to claim 1, wherein the DNA polymerase in said treating step is Deep Vent DNA polymerase.

5. The method according to claim 1, wherein the DNA polymerase in said treating step is Pwo DNA polymerase.

6. The method according to claim 1, wherein the DNA polymerase in said treating step is Pfu DNA polymerase.

7. The method according to claim 1, wherein the polydeoxythymidine-containing oligonucleotide primer further comprises a restriction enzyme cleavage site.

8. The method according to claim 1, wherein the sample of RNA is a mixture of RNA from two or more sources.

9. A method for detection of differential expression of natural antisense messenger RNA (mRNA), comprising:

(a) separately obtaining polyA-mRNA-A molecules from cell population A and polyA-mRNA-B molecules from cell population B;

(b) separately generating by a reverse transcription enzyme a population of single-stranded cDNA-A molecules from polyA-mRNA-A and a population of single-stranded cDNA-B

molecules from polyA-mRNA-B, wherein the polydeoxythymidine containing oligonucleotide primer used to produce the cDNA-B molecules comprises a specific bacteriophage RNA polymerase promoter region close to its 5' terminus;

(c) incubating the combined populations of single-stranded cDNA-A molecules and single-stranded cDNA-B molecules, under conditions allowing hybridization of sense cDNA molecules with antisense cDNA molecules, wherein each single-stranded antisense cDNA molecule that hybridizes has a segment complementary to the sense DNA molecule and hybridizes thereto to form a hybrid molecule with a double-stranded segment;

(d) treating the hybrid molecules with a DNA polymerase having a 5' to 3' polymerase activity and a 3' to 5' exonuclease activity to remove single-stranded non-hybridized segments of the hybrid molecule from 3' to 5' and to extend the double-stranded segment of the hybrid molecule 5' to 3' over an adjacent single-stranded segment as template, thereby forming a double-stranded molecule having the RNA polymerase promoter region close to one terminus;

(e) using the double-stranded molecule as a template for the specific RNA polymerase to produce a population of RNA molecules;

(f) labeling with a first label the RNA molecules produced in step (e);

(g) labeling with a second label as control the polyA-mRNA-A molecules and/or the polyA-mRNA-B molecules of step (a);

(h) mixing labeled RNA molecules from steps (f) and
(g) and hybridizing them to a DNA microarray; and
(i) identifying the genes on the microarray which are preferentially labeled with the labeled RNA molecules of step (f).

10 ~~11~~. The method according to claim 10, wherein the specific bacteriophage polymerase is selected from the group consisting of T7 RNA polymerase, T3 RNA polymerase, and SP6 RNA polymerase.

11 ~~12~~. The method according to claim 10, wherein following step (b) the cDNA-B is modified in order to resist the 3' to 5' exonuclease activity of step (d).

12 ~~13~~. The method according to claim 12, wherein in step (d) the 3' terminus of the cDNA-B is modified.

13 ~~14~~. The method according to claim 12, wherein in step (d) the entire cDNA-B is modified.

14 ~~15~~. The method according to claim 13, wherein 3' terminus of the cDNA-B is modified by addition of a nucleotide analog.

15 ~~16~~. The method according to claim 14, wherein the entire cDNA-B is modified by incorporation of nucleotide analogs.

16 ~~17~~. The method according to claim 10, wherein the

first label of step (f) is Cy3, and the second label of step (g) is Cy5.

17~~18~~. The method according to claim 10, wherein the first label of step (f) is Cy5, and the second label of step (g) is Cy3.

18~~19~~. A method for detection of differential expression of natural antisense messenger RNA (mRNA), comprising:

(a) separately obtaining polyA-mRNA-A molecules from cell population A and polyA-mRNA-B molecules from cell population B;

(b) separately generating by a reverse transcription enzyme a population of single-stranded cDNA-A molecules from polyA-mRNA-A and a population of single-stranded cDNA-B molecules from polyA-mRNA-B, wherein the polydeoxythymidine containing oligonucleotide primer used to produce the cDNA-A molecules comprises close to its 5' terminus a sequence identical to an amplification primer used in step (e) and wherein the polydeoxythymidine containing oligonucleotide primer used to produce the cDNA-B molecules comprises a specific bacteriophage RNA polymerase promoter region close to its 5' terminus;

(c) incubating the combined populations of single-stranded cDNA-A molecules and single-stranded cDNA-B molecules, under conditions allowing hybridization of sense cDNA molecules with antisense cDNA molecules, wherein each single-stranded antisense cDNA molecule that hybridizes has a segment

complementary to the sense DNA molecule and hybridizes thereto to form a hybrid molecule with a double-stranded segment;

(d) treating the hybrid molecules with a DNA polymerase having a 5' to 3' polymerase activity and a 3' to 5' exonuclease activity to remove single-stranded non-hybridized segments of the hybrid molecule from 3' to 5' and to extend the double-stranded segment of the hybrid molecule 5' to 3' over an adjacent single-stranded segment as template, thereby forming a double-stranded molecule having the RNA polymerase promoter region close to one terminus;

(e) amplifying the double-stranded molecule of step (d) using a thermostable polymerase and a first amplification primer identical to the sequence used in step (b) and a second amplification primer identical to the specific bacteriophage RNA polymerase promoter region of step (b);

(f) using the double-stranded molecules so produced as a template for the specific RNA polymerase to produce a population of RNA molecules;

(g) labeling with a first label the RNA molecules produced in step (f);

(h) labeling with a second label as control the polyA-mRNA-A molecules and/or the polyA-mRNA-B molecules of step (a);

(i) mixing labeled RNA molecules from steps (g) and (h) and hybridizing them to a DNA microarray; and

(i) identifying the genes on the microarray which are preferentially labeled with the labeled RNA molecules of step (g).

19₂₀. The method according to claim 19, wherein the specific bacteriophage polymerase is selected from the group consisting of T7 RNA polymerase, T3 RNA polymerase, and SP6 RNA polymerase.

20₂₁. The method according to claim 19, wherein following step (b) the cDNA-B is modified in order to resist the 3' to 5' exonuclease activity of step (d).

21₂₂. The method according to claim 21, wherein in step (d) the 3' terminus of the cDNA-B is modified.

22₂₃. The method according to claim 21, wherein in step (d) the entire cDNA-B is modified.

23₂₄. The method according to claim 22, wherein 3' terminus of the cDNA-B is modified by addition of a nucleotide analog.

24₂₅. The method according to claim 23, wherein the entire cDNA-B is modified by incorporation of nucleotide analogs.

25₂₆. The method according to claim 19, wherein the first label of step (g) is Cy3, and the second label of step (h) is Cy5.

26₂₇. The method according to claim 10, wherein the first label of step (g) is Cy5, and the second label of step (h) is Cy3.